miR-KO System
TALENs and Matched HR Vectors

Cat. # MIR-KO-XXX, MIR-KO-XXXHR-1

User Manual

Grow bacterial stock on receipt
Make plasmid DNA for experiments

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

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I. Introduction

A. Overview of miR-Knockouts (miR-KO)

MicroRNAs (miRNAs) are evolutionarily conserved transcriptional regulators that induce translational repression and mRNA destabilization. Various technical tools have been developed to probe the functions of miRNAs, yet such tools (e.g. antisense oligos) have been limited by low efficiency and specificity. Although genetic knockouts are considered the gold standard for abolishing and studying gene function, the traditional transgenic methodology is less efficient and not readily applicable to most cultured somatic cells or disease models [1,2].

Engineered nucleases cleave chromosomal DNA in a site-specific manner, thus producing a DNA double-strand break (DSB), which in turn triggers either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) to confer gene knockout with high efficiency. To achieve miRNA-specific knockouts, we developed a transcription activator-like effector (TALE) nuclease-based system called miR-KO to target human miRNA genes. TALE-Nucleases have been shown to permanently abolish miRNA function with reduced risk of off-target effects compared to other miRNA targeting strategies, affording researchers a new and exciting set of tools to study miRNA biology.

miR-KOs are transcription activator-like effector (TALE) nucleases that precisely edit specific miRNAs in mammalian cells. We designed miR-TALE-nucleases to cleave within the miRNA seed region (**Fig. 1**). In the absence of HR donor vectors, the

cellular machinery repairs such breaks via non-homologous end joining (NHEJ). This is an error-prone system that typically generates small deletions or insertions (indels) at or near the site of cleavage. Since the seed region (defined as bases 2-8 of the microRNA) directs miRNA binding to its target DNA, indels within the seed region completely abolish miRNA function [3,4].

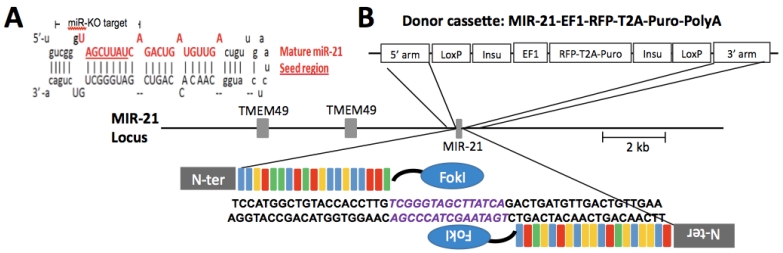


Figure 1. Design of miR-KOs. A) miR-KOs disrupt the miRNA seed region. **B)** Pairing miR-KOs with an HR donor replaces the entire miRNA hairpin structure with an insulated selectable marker cassette.

While the use of miR-KOs alone can successfully abolish miRNA function, screening for bi-allelic indels can be laborious. Due to the small changes seen with indels, many clonal lines have to be established through limited dilution or single-cell sorting techniques, and subsequently genomic DNA is PCR-amplified, cloned into vectors and subjected to genotyping by Sanger sequencing. Since many cells will only have either zero or one alleles modified, tremendous work is often required to obtain bi-allelic indels (Fig. 3. Left Panel).

To facilitate the screening process, one may combine miRNA-specific TALE-nucleases with HR donor vectors (**Fig. 1B**), which enables positive selection and convenient screening of targeted cells (**Fig. 2**). Because NHEJ occurs more frequently than HR donor integration, the majority of cells that undergo HR integration on one allele carry an indel in the miRNA seed region of the second allele. This strategy has been shown to be highly efficient in generating bi-allelic miRNA knockouts (Fig. 3) [3].

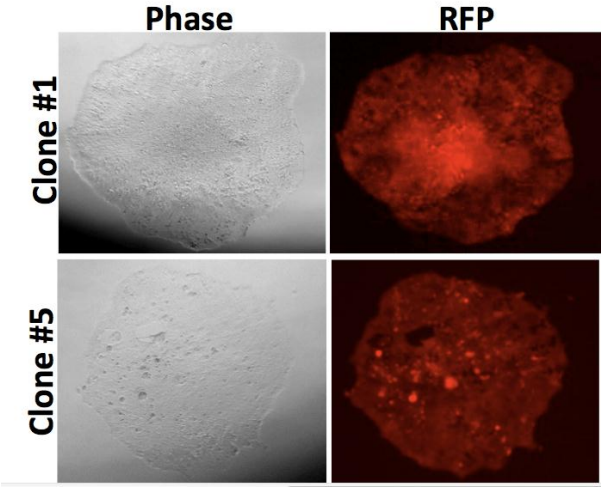


Figure 2. A positive selection strategy reveals puromycin-resistant and RFP-positive single-cell derived colonies, majority of which are double knockouts (i.e. HR event on one allele and indel in seed region of second allele).

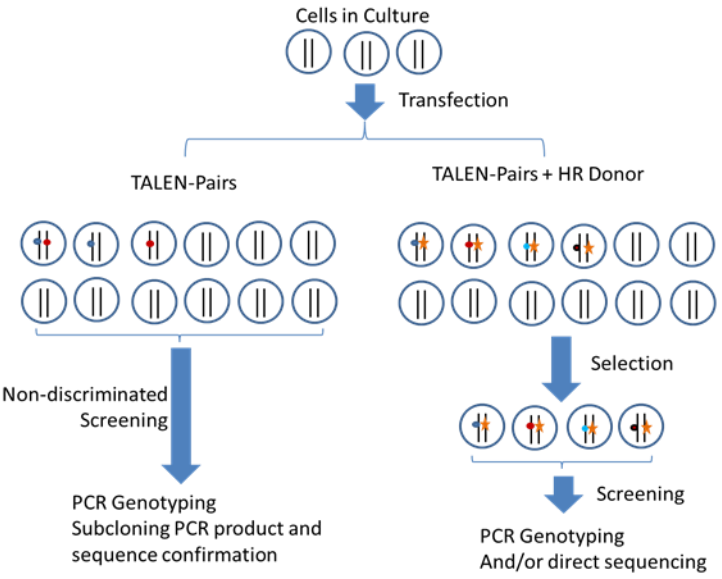


Figure 3. Overview of miR-KO strategies with miR-KOs alone and in combination with an HR donor vector. The HR donor vector enables positive selection, which allows for simple and efficient generation of cells harboring double knockouts.

B. Available miR-KO TALE Nucleases, Corresponding HR Donor Vectors and Stable Cell Lines

miR-KO TALE Nuclease pairs were assembled into CMV-driven expression cassettes using the EZ-TAL Assembly Kit (Cat# GE100A-1; SBI).

A complete list of currently available miR-KO TALE Nucleases, HR Donor Vectors and Complete Kits can be viewed here: http://www.systembio.com/microrna-research/microrna-knockout/mirko-ordering

Product Formats:

Cat#	Description	Format
MIR-KO-XXPA-1	miR-KO hsa-miR-XX TALE Nuclease pair	2 x Bacterial Streaks
MIR-KO-XXHR-1	miR-KO hsa-miR-XX HR Donor Vector (Dual Selection: RFP + Puro)	1 x Bacterial Streak
MIR-KO-XXPA-KIT	miR-KO hsa-miR-XX Complete Kit (includes TALE Nuclease pair and HR Donor Vector)	1 Kit (contains 3 x Bacterial Streaks)
MIR-KO-293-PL	HEK 293 Control Line (RFP-negative, puromycin sensitive)	2x10^6 cells/vial
miR-KO-MIR21	Stable hsa-miR-21 Knock-Out HEK 293 Cell Line (RFP-positive, puromycin	2x10^6 cells/vial

	resistant)	

C. Additional Required Materials

1) Zyppy Endo-Free Plasmid Maxiprep Kit (Zymo Research, Irvine, CA, #D4028)

2) LB Broth (Teknova, #L8000)

3) Carbenicillin (Teknova, #C2130)

4) 1kb Plus Ladder (Life Technologies, #10787-018)

5) Lipofectamine 2000 or 3000 (Life Technologies, #11668-027, #L3000-008)

6) Lonza NucleoFector/Neon Electroporation (for difficult to transfect cells)

D. Related Products

1) EZ-Genotyping Kit (Cat #GE200A-1)

2) EZ-TAL Assembly Kit (Cat #GE1xxA-1)

3) PrecisionX HR Targeting Vectors (Cat #HR1xxPA-1)

4) PrecisionX Cas9 SmartNuclease System (Cat #CAS7xx, CAS9xxA-1)

II. Protocol for using miR-KOs without HR Donor Vector

A. How to use miR-KOs to disrupt miRNA seed region

It is possible to use miR-KOs alone (*i.e.* without an HR donor vector) to generate indels (small insertions or deletions) within the miRNA seed region. Seed regions are responsible for miRNA annealing to their target genes, thus indels within the miRNA seed region completely abolish miRNA function. If there is a highly-efficient transfection protocol available for the specific target cells, one may use miR-KOs alone to induce indels. However, screening for bi-allelic knockouts can still be laborious. In case of difficult-to-transfect cells or genomic loci not easily accessible, we suggest combining miR-KOs with pre-made HR donor vectors (please refer to Table II for available vectors) for enrichment of genetically-modified cells. (For more information, please see Section III, pg.12 for using miR-KOs with HR donor vectors)

B. Propagation of miR-KOs

miR-KOs act as pairs, because the nucleases need to dimerize in order to cut DNA. Left and right miR-KOs are shipped as two separate *E. coli* streaks. We recommend propagating the two plasmids prior to starting the experiments. Bacteria can be grown at 37°C overnight in LB medium containing 50 µg/ml of carbenicillin. After overnight growth, plasmid DNA can be isolated from culture, using an endotoxin-free DNA plasmid kit.

C. Transfection of miR-KOs into target cells

Depending on the cell type being transfected, please choose a transfection protocol that results in maximal transfection efficiencies. For adherent cell lines such as HEK293T, passive transfection methods using lipid-based methods (*e.g.* Lipofectamine 2000/3000, FuGene HD) work well. For other types of cells such as primary, stem, or suspension cells, we suggest transfection using electroporation methods (*e.g.* NucleoFection or Neon) for optimal results.

Note #1: For HEK293T cells, we suggest 0.5-1.0 µg of each miR-KO plasmid (1-2 µg total for miR-KO pair) for efficient cleavage. For other cell types, we suggest optimizing the amounts of plasmid DNA.

Note #2: The plasmids should be mixed well in minimal serum/no antibiotic media plus cationic lipid transfection reagent or electroporation buffer to maximize plasmid delivery.

1) Plate 150,000 to 200,000 cells (e.g. 293T cells) into a single well of a 12-well plate in 1 ml of appropriate growth medium. Include a single well of cells as negative control (which can be non-relevant plasmid DNA).

2) Next day, or when cells are 50-60% confluent, co-transfect target cells with miR-KO pair using a suitable transfection reagent or method following the manufacturer's recommended protocol for 12-well plates.

3) Allow at least 12 hours before changing transfection media to complete growth media.

4) Assay for miR-KO functionality at 48-96 hours after co-transfection. General cutting efficiency of miR-KOs can be measured by Surveyor Nuclease Assay. For more thorough analysis of targeted cells, single cell colonies will need to be derived from the initial transfection pool (please see Section D below).

D. Screening for indels in miRNA seed region

The percentage of alleles carrying indels within the miRNA seed region can be assessed with a commercial genotyping kit (*e.g.* EZ-Genotyping™ Kit; SBI, Cat# GE200A-1) or by High Resolution Melt Analysis (HRMA) analysis [4], keeping in mind that TALE-nucleases typically cut the mammalian genome with a frequency of 0.5-40%. The efficiency varies greatly depending on the cell type(s) being targeted as well as the transfection method employed. After transfection, isolating bi-allelic knockouts requires single cell-derivation via serial dilution approaches (http://csmedia2.corning.com/LifeSciences/Media/pdf/Single_cell_cloning_protocol.pdf) or single-cell sorting, followed by propagation of the clone and finally screening of clones via PCR and Sanger sequencing. While it is possible to screen for these knockouts via genotyping of single-cell-derived clones, the process can be laborious and time-consuming. Thus we recommend combining miR-KOs with an HR donor vector (pre-made and available for each miR TALE-nuclease pair) to greatly simplify screening for bi-allelic knockouts.

III. Protocol for use of miR-KOs with HR Donor Vector

A. How to use miR-KOs with miR-specific HR vector to remove entire miRNA hairpin

Combining miR-KOs with a pre-made HR donor vector is a convenient way to generate miRNA double knockouts. The HR vector arms are designed to delete the entire miRNA stem-loop structure (70~200 bp). The HR donor vector includes an insulated cassette for RFP and puromycin markers to allow positive selection by drug treatment and/or fluorescent-activated cell sorting. This approach has been shown to be highly efficient for generating bi-allelic miRNA knockouts [3].

B. Transfection of miR-KOs into target cells

Depending on the cell type being transfected, please choose a transfection protocol that results in maximal transfection efficiencies. For adherent cell lines such as HEK293T, passive transfection methods using cationic lipid-based methods (*e.g.* Lipofectamine 2000/3000, FuGene HD) work well. For other types of cells such as primary, stem or suspension cells, we suggest transfection using electroporation methods (NucleoFection or Neon) for optimal results.

Note #1: For HEK293T cells, we suggest 0.5-1.0 µg of each miR-KO plasmid (1-2 µg total for miR-KO pair) and 1 ug of the HR donor vector. For other cell types, we suggest optimizing the amounts of plasmid DNA.

Note #2:The plasmids should be mixed well in minimal serum/no antibiotic media plus cationic lipid transfection reagent or electroporation buffer to maximize efficiency of delivery.

Note #3: For selection of target cells, we recommend testing different concentrations of puromycin on untransfected cells to determine the optimal concentration, which should kill ~90-100% of cells within 48-72 hours after drug administration.

1) Plate 150,000 to 200,000 cells (e.g. 293T cells) into a single well of a 12-well plate in 1 ml of appropriate growth medium. Include a single well of cells as negative control (which can be non-relevant plasmid DNA).

2) Next day, or when cells are 50-60% confluent, co-transfect target cells with miR-KO pair using a suitable transfection reagent or method following the manufacturer's recommended protocol for 12-well plates.

3) Allow at least 12 hours before changing transfection media to complete growth media.

4) 48-96 hours after co-transfection, sort cells using FACS or start Puromycin selection (please see Section C below).

C. Selection for HR events

Select for HR events by puromycin or by FACS-based sorting for RFP.

When using selection by puromycin, grow single cell under puromycin pressure for a minimum of 20 days to obtain enough cells for genotyping and storage.

Using FACS-based sorting for the RFP marker, assay for positive HR events using approximately 25,000 positive sorting events as a minimum for genotyping analysis (please see Section D below) and assessing the percent of cells that have undergone homologous recombination.

Note: The percentage of *alleles* carrying indels within the miRNA seed region can be assessed with a commercial genotyping kit (*e.g.* EZ-Genotyping™ Kit; SBI, Cat# GE200A-1) or High Resolution Melt Analysis (HRMA) analysis. HR efficiency can be determined via FACS analysis as % of RFP positive cells.

D. Genotyping

I) Genotyping for HR event via junction PCR

Genotyping of selected cells can be done by PCR of genomic DNA-insert junctions at 5' and/or 3' ends of HR site (Figure 4). Design PCR primer pairs with one of the primers in the genomic

DNA region and the other located in the HR vector [3]. In this way, only positive

HR events will result in correct PCR products, whereas random integration or no-HR cells will not produce PCR products. Figure 5 shows the positive and negative clones using EZ-Genotyping™ Kit to confirm HR events.

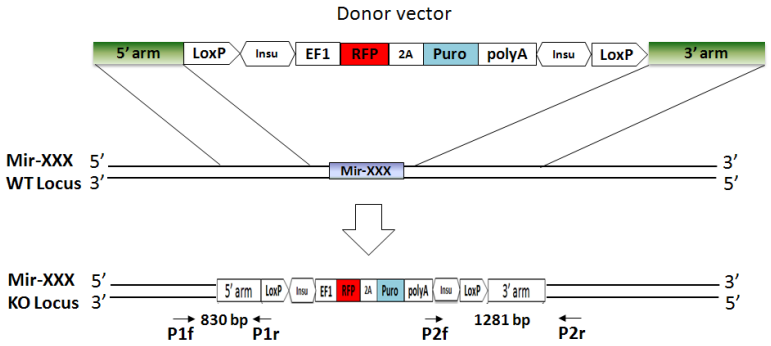


Figure 4. Schematics of primer location to confirm HR donor integration

The primers of P1f and P2r are located outside the 5' or 3' arm region, whereas the P1r and P2f are donor plasmid specific primers.

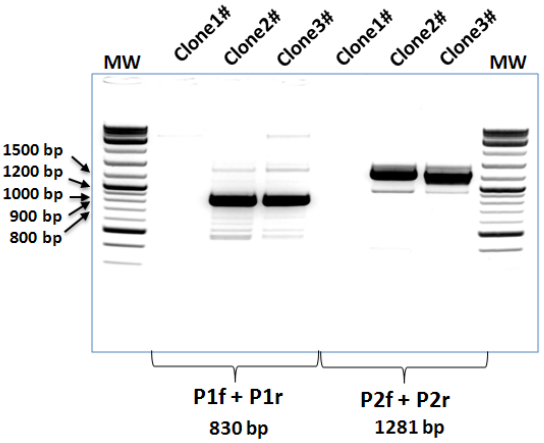
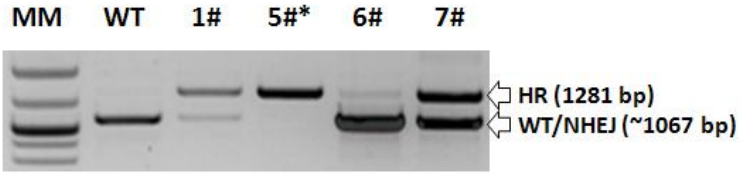


Figure 5. Genotyping by junction PCR analysis using EZ-Genotyping Kit. Amplification of genomic DNA from Clone #1 shows no predicted PCR products indicating no HR events occurred (or random integration of donor), whereas Clone #2 and Clone #3 exhibit correct junction PCR products, confirming HR integration

II) Genotyping for double knockouts

To completely knock out a miRNA gene in diploid cells, both miRNA alleles must be disrupted. Because NHEJ occurs more frequently than HR events, most selected cells will be double knockouts, with the second allele being disrupted by NHEJ-mediated mutation (**Fig. 3**). In some cases, both miRNA alleles are removed by an HR event (**Fig. 6A, clone #5**). Genotyping PCR can be used to detect HR events. Clones that reveal one HR-modified allele can be further tested by amplifying and sequencing of the miRNA seed region (**Fig. 6B**).

A



B

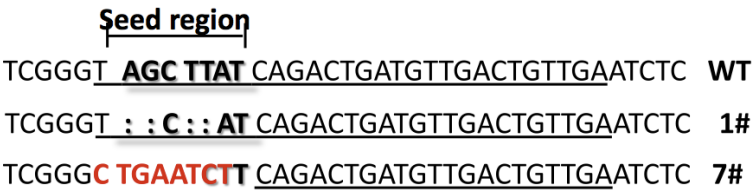

Seed region
TCGGGT AGC TTAT CAGACTGATGTTGACTGTTGAATCTC WT
TCGGGT : : C : : AT CAGACTGATGTTGACTGTTGAATCTC 1#
TCGGGC TGAATCTT CAGACTGATGTTGACTGTTGAATCTC 7#

Figure 6. Genotyping of selected clones. A) PCR to detect HR integration in one or both alleles **B)** Clones that display one HR event typically display mutated seed regions in the other allele.

IV. Validation Data for miR-KOs

A. High frequency of double knockouts

miR-KOs combined with HR donor vectors have been shown to be highly efficient in generating double miRNA knockouts [3]. For example, a miR-KO strategy against human miR-21 in HEK293T cells resulted in 30 puromycin-resistant lines out of 96 single cell-derived clones. Subsequent PCR-based genotyping of 23 successful PCR amplifications revealed that ~96% (22/23) were mono-allelic (i.e. one allele with HR and other with NHEJ or WT)

and ~4% (1/23) were bi-allelic (e.g. both alleles undergone HR) for HR-induced miR-21 deletion. Furthermore, sequencing of PCR products spanning the targeted seed region of miR-21 revealed that 91% (10/11) were NHEJ-modified. Taken together, these results show a 87% bi-allelic modification rate (20 out of 23 clones) when the miR-KOs are combined with HR donor vector [3].

B. Permanent and complete knockouts

To confirm complete loss of miRNA expression, we quantified miR-21 expression in three independent miR-21 double knockouts. Clone #1 and #7 carry one deletion of the miR-21 hairpin structure (via HR) and one indel within the seed region (via NHEJ); clone #5 carries bi-allelic deletions of the hairpin structure (bi-allelic HR). As shown in **Fig.6**, we found complete abolishment of miR-21 expression in all three cell lines.

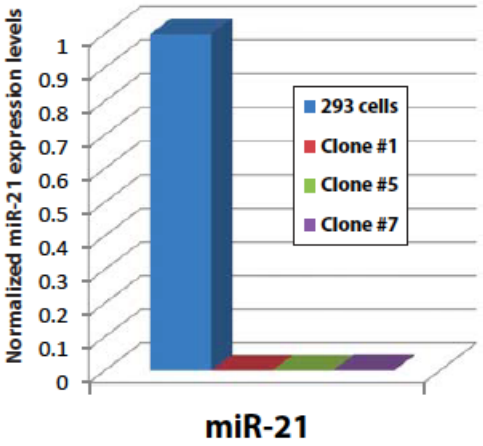


Figure 6. Quantitative PCR analysis reveals complete ablation of miR-21 expression in bi-allelic miR-21 knockouts.

V. References

[1] D.M. Patrick, R.L. Montgomery, X. Qi, S. Obad, S. Kauppinen, J.A. Hill, E. van Rooij, E.N. Olson, Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice, J Clin Invest 120 (2010) 3912-3916.

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[3] C. Uhde-Stone, N. Sarkar, T. Antes, N. Otoc, Y. Kim, Y.J. Jiang, B. Lu, A TALEN-based strategy for efficient bi-allelic miRNA ablation in human cells, RNA (2014).

[4] R. Hu, J. Wallace, T.J. Dahlem, D.J. Grunwald, R.M. O'Connell, Targeting Human MicroRNA Genes Using Engineered Tal-Effector Nucleases (TALENs), PLoS One 8 (2013) e63074.

VI. Technical Support

For more information about SBI products or to download manuals in PDF format, please visit our website:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

tech@systembio.com

650-968-2200

VII. Licensing and Warranty Statement

System Biosciences ("SBI") warrants the product meets the specifications described in this manual. SBI cannot guarantee the biological function and cleavage efficiency of miR-KOs in a particular cell line.

HR donor vector sequences may vary slightly from annotated Genbank sequences due to SNPs and satellite repeat variations. As microRNAs are non-coding, such variations may not interfere with the biological function nor with recombination of the HR donor vector.

SBI is committed to providing our customers with high-quality products. If it is proven to the satisfaction of SBI that the Product fails to meet the specifications outlined in this manual, SBI will replace the Product or provide the purchaser with a credit. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product. SBI's liability is expressly limited to replacement of Product or a credit limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.